BioFactors 11 (2000) 171–177
IOS Press

D2

Phytate prevents tissue calcifications in female rats ⟹ describe ingestión de fitato

F. Grases *, R.M. Prieto, B.M. Simonet and J.G. March
Laboratory of Renal Lithiasis Research, University of Balearic Islands, Palma de Mallorca, Spain

NO aplicación tópica.

Received 28 December 1999
Revised 10 January 2000
Accepted 7 February 2000

Abstract. The AIN-76 A, a purified rodent diet, has a propensity to cause kidney calcifications in female rats which is not observed with non-purified rodent diets, suggesting a nutritional factor that avoids these calcifications. One candidate is phytate, which inhibits crystallisation of calcium salts and is practically absent in purified diets. Therefore, the effects on calcification of kidney tissue of phytate addition to the AIN-76 A diet using female Wistar rats were studied. The rats were assigned to three groups: AIN-76 A, AIN-76 A + 1% phytate and standard nonpurified chow. Urinary phytate of the AIN-76 A fed group was undetectable. Urinary phytate of AIN-76 A + 1% phytate and standard fed groups did not differ and was significantly higher than in the AIN-76 A group. The concentrations of calcium and phosporus in kidneys were greater in the AIN-76 A group than in AIN-76 A + 1% phytate and standard groups. Only rats of the AIN-76 A group displayed mineral deposits at the corticomedullary junction. These findings demonstrated that the absence of phytate in the AIN-76 A diet is one of the causes of renal calcification in female rats.

oral

1. Introduction

The majority of the human biological fluids are supersaturated with some compounds. Blood and interstitial liquid for instance are supersaturated with respect to calcium phosphate (hydroxyapatite). Urine is always calcium oxalate supersaturated and, depending on its pH value, is also supersaturated with respect to uric acid (pH < 5.5) or calcium phosphate (pH > 6.0) [4]. Physiologically, crystallisation only takes place in controlled situations like in the formation of bone and teeth. Nevertheless, uncontrolled pathological crystallisation such as calculi formation (renal, biliar, sublingual), calcification of atheroms, tissue calcification associated with cancer, etc. is also frequent. In fact, crystallisation does not take place indiscriminately in all human fluids because it does not depend exclusively on thermodynamic factors but also on kinetic factors. There are three main aspects to explain pathological crystallisation: supersaturation higher than usual (thermodynamic factor), the presence of heterogeneous nuclei (crystallisation inducers, kinetic factor), and/or deficit of crystallisation inhibitors (kinetic factor). Under physiological conditions, the crystallisation inhibitors at adequate concentrations may prevent the development of solid concretions by delaying the crystallisation of supersaturated substances, until a readjustment of concentration in the corresponding fluids is achieved.

*Correspondence to: Dr Felix Grases, Laboratory of Renal Lithiasis Research, Edifici Mateu Orfila, University of Balearic Islands, Ctr. of Valldemossa, km 7.5, E-07071 Palma de Mallorca, Spain. Tel.: +34 971 17 32 57; Fax: +34 971 17 34 26; E-mail: dqufgf0@ps.uib.es.

Recently, it has been demonstrated that phytate (myo-inositol hexaphosphate), present in blood, urine, interstitial and intracellular fluids [1,10] inhibits crystallisation of calcium salts such as oxalate and phosphate [5,6,8]. In a nephrolithiasis animal model using ethylene glycol, phytate was found to significantly reduce the number of calcifications on the papillary tips and the total calcium content of the papillary tissue [7]. Phytate may also participate in endochondral ossification impeding the mineralization of vesicles, a process believed to be regulated by enzymatic phytate hydrolysis [2].

From the beginning, there have been many problems with the AIN-76 A purified rodent diet, the major one being their propensity to cause kidney calcification in female rats [14–16], whereas commercial non-purified diets generally are less nephrocalcinogenic [11,13]. In non-purified cereal-based diets an important part of the phosphorus content represents phytate, which in purified diets phytate is practically absent. In the present paper, therefore, the effect of phytate addition to the AIN-76 A diet on calcification of kidney tissue are studied. ← oral.

2. Materials and methods

2.1. Animals and diets

Female Wistar rats (21 days-old) from Harlan Ibérica S.A. (Barcelona, Spain) were acclimated to our animal house in the course of 7 d and kept on standard non-purified diet and tap water. The rats were housed two animals per cage at a temperature of 23 ± 1°C and relative humidity of 50% with a 12-h on-off light cycle. The animals were assigned randomly to three groups of 12 rats.

The diets used were AIN-76 A (Harlan Tekland, WI, USA), a purified diet in which phytate is practically absent, an AIN-76 A modified diet (AIN-76 A + 1% phytate), to which phytate dodecasodium salt from corn (Sigma-Aldrich, Madrid, Spain) was added to obtain 1 g/100 g (Harlan-Tekland, WI, USA) (Table 1) and the standard non-purified diet pellets (UAR A03, Panlab s.l., Barcelona, Spain; water 10%; dry wt., 26.7% proteins, 56.5% carbohydrate, 5.7% lipids, 4.5% cellulose and 6.5% ashes). Each experimental group (AIN-76 A, AIN-76 A + 1% phytate and standard non-purified diet) were fed one of the different diets for 12 wk. Representative analyses of calcium, magnesium, phosphorus, phytate and water in AIN-76 A, AIN-76 A enriched 1% phytate and standard nonpurified diets are shown in Table 2.

Table 1
Composition of AIN-76 A and AIN-76 A enriched with 1% phytate purified diets

	AIN 76 purified diet (g/kg)	AIN 76 A + phytate (g/kg)
Casein, high protein	200.0	200.0
D-L Methionine	3.0	3.0
Sucrose	499.99	489.99
Corn starch	150.0	150.0
Corn oil	50.0	50.0
Fibre (cellulose)	50.0	50.0
Mineral mix, AIN 76	35.0	35.0
Vitamin mix, AIN 76	10.0	10.0
Choline bitartrate	2.0	2.0
Ethoxyquin (antioxidant)	0.01	0.01
Phytate dodecasodium salt	–	18.42

Table 2
Calcium, magnesium, phosphorus and phytate in AIN-76 A, AIN-76 A enriched with 1% phytate and standard nonpurified diet UAR A03

	Diets		
	AIN-76 A	AIN-76 A 1% phytate	UAR A03
Calcium (mmol/100 g)	9.25	10.47	22.27
Phosphorus* (mmol/100 g)	21.96	22.29	14.32
Ca : P molar ratio	0.42	0.47	1.55
Magnesium (mmol/100 g)	1.87	1.96	6.95
Phytate (mmol/100 g)	undetectable	1.70	1.23
Water (g/100 g)	5.5	6	10

*Excluded phosphorus from phytate.

The procedures used in this experiment were made according the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

2.2. *Chemical analyses and pathological examination*

Regularly (every 4 weeks) 24-h urine was collected by housing the rats of the respective groups in different metabolic cages (Tecniplast Gazzada s.a.r.l., Italy). Urine samples were analysed for calcium, magnesium and phosphorous by ICP atomic emission spectrometry and for phytate following the procedure detailed below.

On the final day of the experiment all animals were anaesthetised with pentobarbital (50 mg/kg, i.p.), sacrificed, and kidneys were removed. One half of each kidney was lyophilised (Cryodos, Telstar, Barcelona, Spain) to constant weight and ashed in a muffle furnace at 500°C for 24 h until white ash was reached. The ash was dissolved in HCl (1 mol/l) and the concentrations of calcium, magnesium and phosphorous were determined by ICP atomic emission spectrometry. The other half of the kidney was fixed in formaldehyde. Slices of the kidneys were embedded in paraffin, hematoxylin-eosin stained and examined by light microscopy (Olympus BH II, Japan).

2.3. *Phytate determination*

Alimentary and urinary phytate were determined essentially according to [10]. Briefly a 0.25 g diet sample extrated with 50 ml of HCl (0.5 mol/l) or a fresh acidified urine sample were clarified by passage trough a charcoal column. Five ml of the clarified sample were applied to an anionic exchange column (AG 1 × 8, 200–400 mesh. BioRad, Hercules, CA, USA), the column was washed with HCl (0.05 mol/l), and phytate was eluted with 5.0 ml H_2SO_4 (2 mol/l). Contamination with phosphate was determined in an aliquot, while phytate-bound phosphate was determined after hydrolysis (120°C during 36 h). Phosphate determination was carried out molybdenum blue formation followed by a liquid-liquid extraction in a small volume of ethylacetate with Adogen 464 (methyltrialkil (C_8–C_{10}) ammonium chloride) (Sigma-Aldrich, Madrid, Spain). After phase separation, the absorbance at 715 nm was measured. All chemical reagents used were of analytical grade. The detection limit of phytate was 0.1 mg/l.

2.4. *Statistics*

Values in the tables and figures are expressed as mean ± SE. One-way ANOVA was used to calculate significance of differences between groups. The Student *t*-test was used to assess differences of means.

The SPSS for the Windows program was used for statistical computations. A probability of $p < 0.05$ was used for assessing statistical significance.

3. Results

Urinary excretion of calcium, magnesium, phosphorus and phytate was determined at regular intervals (Fig. 1 and Table 3). Urinary excretion of phytate did not differ significatly between the AIN-76 A + 1% phytate and standard groups, whereas in the group fed with AIN-76 A diet phytate was practically undetectable. No important differences in urinary excretion of calcium, magnesium and phosphorous were observed between the three groups.

The female rats fed the AIN-76 A diet had highly anomalous calcium amounts in their kidneys when compared with standard female rats, whereas this amount was significantly reduced in female rats fed with the AIN-76 A enriched diet with 1% phytate (Fig. 2). The phosphorus content in the kidneys of rats fed the AIN-76 A diet was also higher than that of standard and AIN-76 A + 1% phytate diet-fed rats. Only female rats fed the AIN-76 A diet consistently displayed mineral deposits at the corticomedulary junction (Fig. 3). In fact in rats fed AIN-76 A + 1% phytate, calcifications only were observed in a few cases and in areas of low extension when compared with the ones detected in female rats fed AIN-76 A diet.

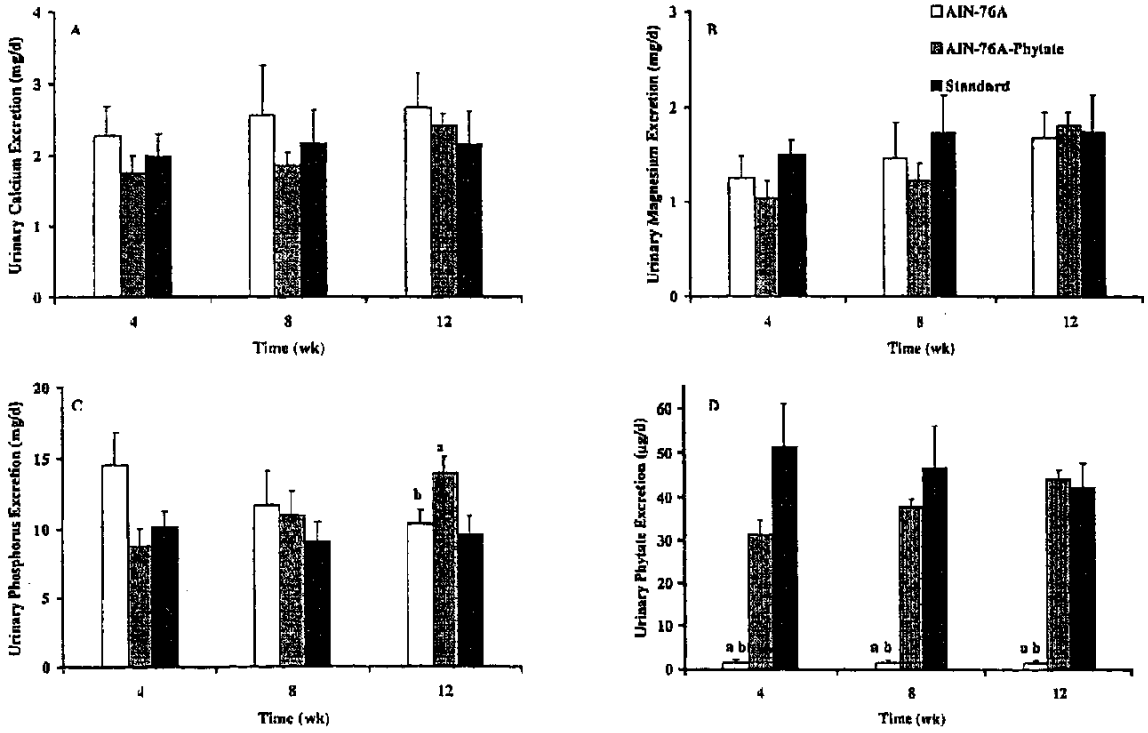

Fig. 1. Urinary excretion of (A) calcium, (B) magnesium, (C) phosphorus and (D) phytate of rats fed the AIN-76 A, the AIN-76 A + 1% phytate diet and a non-purified diet at 4, 8 and 12 weeks. Dates are means ± SE of 12 rats per group. Student t-test was used to determine significant differences between means. [a] $p < 0.05$ vs. standard group. [b] $p < 0.05$ vs. AIN-76 A + 1% phytate group.

Table 3

Rat weight, daily ingested food, urinary volume, calcium, magnesium, phosphorus and phytate concentrations in urine of rats fed the AIN-76 A diet, the AIN-76 A + 1% phytate diet and the standard non-purified diet UAR A03 at 4, 8 and 12 weeks

Group	AIN-76 A	AIN-76 A 1% phytate	Standard
		Time fed 4 wk	
Animal weight (g)	158 ± 4	163 ± 2	163 ± 2
Ingested food (g/d)	12.0 ± 0.6[a]	13.7 ± 0.6	14.5 ± 0.6
Volume (ml)	14.3 ± 2.6	11.5 ± 1.3	15.3 ± 1.7
Calcium (mmol/l)	3.99 ± 0.12[a]	3.77 ± 0.19	3.21 ± 0.18
Magnesium (mmol/l)	3.60 ± 0.12[a]	3.68 ± 0.19	4.13 ± 0.10
Phosphorus (mmol/l)	33.90 ± 2.90[a,b]	24.25 ± 1.84	21.64 ± 0.84
Phytate (mg/l)	<0.1[a,b]	2.75 ± 0.04	3.26 ± 0.26
		Time fed 8 wk	
Animal weight (g)	206 ± 6	211 ± 4	218 ± 3
Ingested food (g/d)	13.7 ± 0.6[a]	14.4 ± 0.4[a]	16.6 ± 0.7
Volume (ml)	14.7 ± 3.8	13.1 ± 4.4	15.3 ± 3.0
Calcium (mmol/l)	4.26 ± 0.10[a]	3.66 ± 0.22	3.51 ± 0.16
Magnesium (mmol/l)	4.04 ± 0.15	3.83 ± 0.08	4.49 ± 0.19
Phosphorus (mmol/l)	27.09 ± 2.68[a]	26.52 ± 1.58	20.39 ± 1.35
Phytate (mg/l)	<0.1[a,b]	2.76 ± 0.29	3.02 ± 0.09
		Time fed 12 wk	
Animal weight (g)	228 ± 7	236 ± 4	240 ± 5
Ingested food (g/d)	13.9 ± 0.6[b]	15.6 ± 0.3	16.2 ± 0.9
Volume (ml)	16.7 ± 2.8	18.0 ± 1.3	16.1 ± 1.5
Calcium (mmol/l)	3.87 ± 0.17[a]	3.38 ± 0.24	3.28 ± 0.17
Magnesium (mmol/l)	4.17 ± 0.05	4.14 ± 0.07	4.48 ± 0.15
Phosphorus (mmol/l)	24.29 ± 3.45	26.29 ± 2.22[a]	19.39 ± 1.03
Phytate (mg/l)	<0.1[a,b]	2.51 ± 0.14	2.70 ± 0.13

[a] $p < 0.05$ vs. standard. [b] $p < 0.05$ vs. AIN-76 A + 1% phytate. Dates are means ± SE of 12 rats per group.

4. Discussion and conclusion

As expected, rats fed the AIN-76 A diet exhibited important corticomedullary calcifications and excreted undetectable amounts of phytate. These results totally agree with previous studies demonstrating that the urinary phytate levels are related to its oral intake [9]. As can be seen in Table 2, the AIN-76 A diet and the phytate-enriched one approximately had the same Ca : P molar ratio and the ratio of the urinary output to intake of these minerals was similar among these two diets. The addition of 1 g/100 g phytate restored the normal urinary levels of this compound, the excretion then being similar to those found in the standard fed group. Without any important changes in calcium, magnesium and phosphate excretions, a significant decrease of calcium accumulation in kidneys of female rats was observed. Moreover, phosphate accumulations in kidneys was lower and calcifications were practically not observed. All these findings clearly demonstrate that the ingestion of phytate efficiently prevents the pathological calcifications of kidney tissue. A low consumption of phytate may thus cause a urinary deficit of this

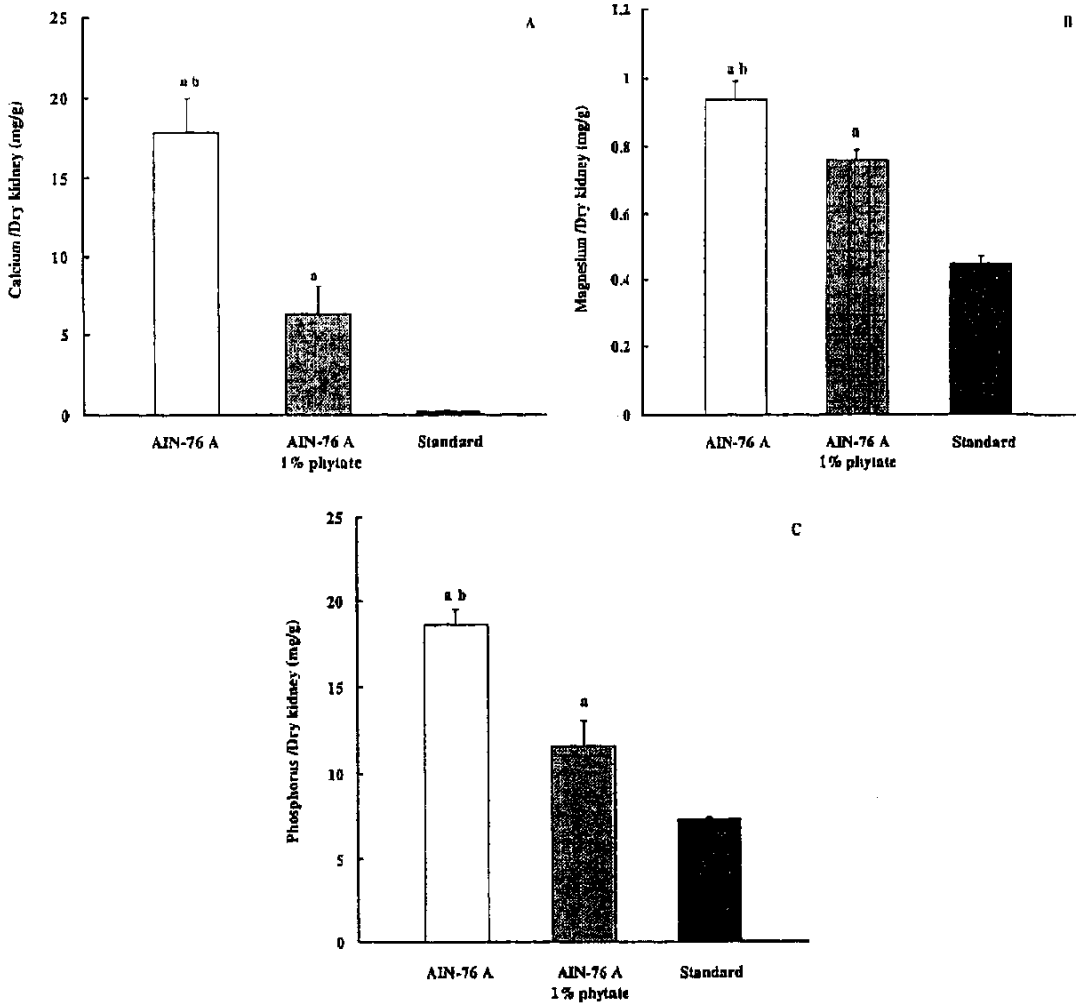

Fig. 2. (A) Calcium, (B) magnesium and (C) phosphorus concentration (mg/g dry weight) in kidneys of rats fed the AIN-76 A diet, the AIN-76 A + 1% phytate diet and non-purified diet. Dates are means ± SE of 12 rats per group. Student t-test was used to determine significant differences between means. $^{a}p < 0.05$ vs. standard group. $^{b}p < 0.05$ vs. AIN-76 A + 1% phytate group.

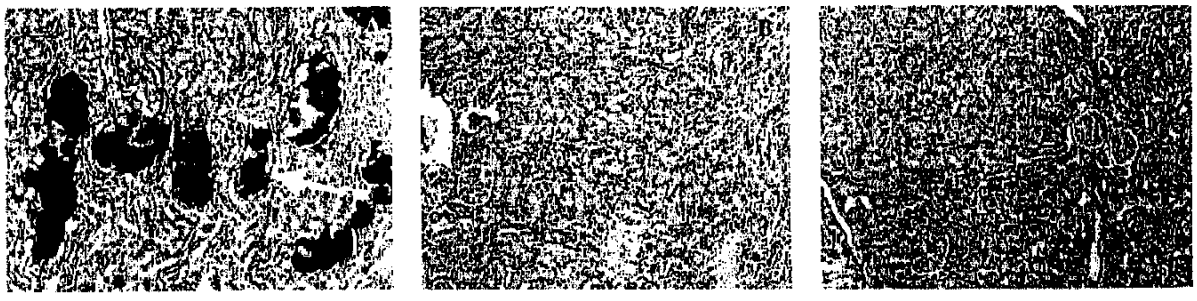

Fig. 3. Longitudinal kidneys sections from rats fed (A) AIN-76 A diet, (B) AIN-76 A + 1% phytate and (C) standard non-purified diet. Hematoxylin-eosin stained section. Magnification of 50×. Arrow shows extensive calcification throughout the corticomedullary region.

crystallisation inhibitor and, in consequence, may increase the risk to develop calcium stones. In fact, the low incidence of renal stones in some ethnic groups has been attributed to the high consum of phytate rich meals [12] and the ingestion of a phytate supplement significantly reduced the risk to develop calcium stones in humans [3].

Acknowledgements

The authors gratefully acknowledge the histological advice of Dr J.J. Torres, Department of Pathology, Hospital of Manacor (Spain) and financial support from *Dirección General de Enseñanza Superior e Investigación Científica* (Grant number PM97-0040).

References

[1] C.M. Bunce, P.J. French, P. Allen, J.C. Mountford, B. Moor, M.F. Greaves, R.H. Michell and G. Brown, Comparison of the levels of inositol metabolites in transformed haematopoietic cells and their normal counterparts, *Biochemical Journal (London)* **289** (1993), 667–673.
[2] J.J. Caffrey, K. Hidaka, M. Matsuda, M. Hirata and S.B. Shears, The human and rat forms a multiple inositol polyphosphate phosphatase: functional homology with histidine acid phosphatase up-regulated during endochondral ossification, *FEBS Letters* **442** (1999), 99–104.
[3] A. Conte, P. Pizá, A. García-Raja, F. Grases, A. Costa-Bauza and R.M. Prieto, Test of urinary lithogen risk: usefulness in the evaluation of renal lithiasis treatment using crystallisation inhibitors (citrate and phytate), *Archivos Españoles de Urologia* **52** (1999), 94–99.
[4] B. Finlayson, Physiochemical aspects of urolithiasis, *Kidney International* 1 (1978), 344–360.
[5] F. Grases, L. Garcia-Ferragut and A. Costa-Bauza, Study of the early stages of renal stone formation: experimental model using urothelium of pig urinary bladder, *Urological Research* 24 (1996), 305–311.
[6] F. Grases, L. Garcia-Ferragut, A. Costa-Bauza and J.G. March, Study of the effects of different substances on the early stages of papillary stone formation, *Nephron* 73 (1996), 561–568.
[7] F. Grases, R. García-Gonzalez, J.J. Torres and A. Llobera, Effects of phytate on renal stone formation in rats, *Scandinavian Journal of Urology and Nephrology* 32 (1998), 262–265.
[8] F. Grases and A. Llobera, Experimental model to study sedimentary kidney stones, *Micron* 29 (1998), 105–111.
[9] F. Grases, B.M. Simonet, J.G. March and R.M. Prieto, Inositol hexakisphosphate in urine: the relationship between oral intake and urinary excretion, *British Journal of Urology International* 85 (2000), 138–142.
[10] J.G. March, B.M. Simonet, F. Grases and A. Salvador, Indirect determination of phytate in urine, *Analytica Chimica Acta* **367** (1998), 63–68.
[11] Y.W.H.M. Mars, A.G. Lemmens and A.C. Beynen, Dietary phosphorus and nephrocalcinosis in female rats, *Nutrition Reports International* 38 (1988), 249–258.
[12] M. Modlin, Urinary phosphorylated inositols and renal stone, *Lancet* 22 (1980), 1113–1114.
[13] A.K.J. Ral-Modhefer, J.C. Atherton, H.O. Garland, H.J. Singh and J. Walker, Kidney function in rats with corticomedullary nephrocalcinosis: effects of alterations in dietary calcium and magnesium, *Journal of Physiology* 380 (1986), 405–414.
[14] P.G. Reeves, F.H. Nielsen and G.C. Fahey Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76 A rodent diet, *Journal of Nutrition* 123 (1993), 1939–1951.
[15] P.G. Reeves, K.L. Rossow and J. Lindlauf, Development and testing of the AIN-93 purified diets for rodents: results on growth, kidney calcification and bone mineralization in rats and mice, *Journal of Nutrition* 123 (1993), 1923–1931.
[16] J. Ritskes-Hoitinga, A.G. Lemmensa, L.H.J.C. Danse and A.C. Beynen, Phosphorus-induced nephrocalcinosis and kidney function in female rats, *Journal of Nutrition* 119 (1989), 1423–1431.